ANNALS OF SURGERY
Vol. 231, No. 2, 253–261

Injury Induces Deficient Interleukin-12 Production, But Interleukin-12 Therapy After Injury Restores Resistance to Infection

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Objective

To assess at serial intervals the production of interleukin-12 (IL-12) by monocytes/macrophages from the peripheral blood of injured patients and control subjects, and using a mouse model to confirm human findings and explore the effectiveness of low-dose IL-12 therapy in restoring resistance to infection after injury.

Summary Background Data

Serious injury is associated with loss of function of the T helper 1 lymphocyte phenotype, but little is known about IL-12 production in injured patients. The authors previously reported that early, moderate-dose IL-12 therapy in a mouse model of burn injury restored resistance to a later infectious challenge (cecal ligation and puncture, CLP). However, the efficacy of clinically relevant low-dose IL-12 therapy carried out to or beyond the time of septic challenge remains to be tested.

Methods

Peripheral blood mononuclear cells (PBMCs) and adherent cells were obtained from 27 patients with major burns or traumatic injury and 18 healthy persons and were studied at serial intervals for IL-12 production stimulated by bacterial lipopolysaccharide (LPS). PBMCs from 18 of the same patients were studied for IL-10 production as well. IL-12 production by adherent cells from the spleens of burn or sham burn mice was studied at serial intervals after injury to confirm the human findings. Low-dose IL-12 or vehicle was given every other day to groups of burn and sham burn mice, which were then challenged with CLP on day 10, and survival was determined. Finally, spleens were harvested from burn or sham burn animals receiving low-dose IL-12 or vehicle after CLP. After splenic cellularity was determined by hemocytometer, splenocytes were cultured and production of tumor necrosis factor-alpha, interferon-gamma, and IL-10 were assessed by immunoassay.

Results

Adherent cells from patients' PBMCs produced significantly less IL-12 than normal PBMCs after injury, reaching a nadir 8 to 14 days after injury. Stimulation of whole PBMCs by LPS indicated that at 8 to 14 days after injury, IL-12 production by PBMCs was significantly lower and IL-10 production was significantly higher than that of PBMCs from healthy persons. Low-dose IL-12 therapy significantly increased survival after CLP. Splenocytes from burn mice treated with IL-12 had significantly increased production of TNF-α and IF-γ, both before and after CLP, when compared with vehicle-treated burn animals. IL-10 production by burn splenocytes remained high after IL-12 treatment. Splenic cellularity increased after IL-12 treatment in burn mice.

Conclusion

The capacity to produce IL-12 by adherent cells of the monocyte/macrophage lineage is significantly reduced after serious injury in humans and in a mouse burn model. In humans, there is a reciprocal relation between diminished IL-12 production and increased IL-10 production at approximately 1 week after injury. Low-dose IL-12 therapy in the mouse burn model markedly increased survival after a septic challenge, even when treatment was carried beyond the onset of sepsis. Low-dose IL-12 treatment in the mouse increased production of proinflammatory mediators important in host defense and at the same time maintained or increased production of IL-10, an important antiinflammatory cytokine.

Serious injury induces diminished resistance to infection, and sepsis is a leading cause of death in injured patients who survive initial resuscitation. Attempting to explain this phenomenon, numerous investigators over the past two decades have documented an association between major traumatic or thermal injury and perturbations of both the innate and adaptive immune systems.[1]

The cell type responsible for initiating most adaptive immune responses is the T lymphocyte, and observations from several laboratories, including our own, have demonstrated that injury induces alterations in T-cell function.[2–6] More recently, we and others have presented evidence that in patients and in animal models, serious injury is followed by loss of function of the T helper 1 (Th1) lymphocyte subset with preservation of T helper 2 (Th2) function, as indicated by cytokine production studied in vitro.[4–10] These in vitro observations have been complemented by the in vivo demonstration that major injury is associated with loss of production of antibody isotypes induced by Th1 cells with preservation of isotypes induced by Th2 cells.[11] Because Th1 cells are principal regulators of cell-mediated immune responses and of the production of complement fixing antibodies,[12] depressed function of this lymphocyte subset might be expected to be detrimental to the ability to resist infection. Indeed, in animal models of injury, therapeutic regimens designed to increase Th1 function have been associated with improved survival after a septic challenge.[13–15]

Naive T cells can be induced to express the Th1 phenotype by exposure to the cytokine interleukin-12 (IL-12), whereas exposure to IL-4 induces the Th2 phenotype.[12] Major injury has been shown to result in increased IL-4 production in both humans and experimental animals,[4,5,7–9] and we have previously reported diminished IL-12 production by monocyte/macrophages in a murine burn model by day 10 after injury.[7] However, little is known about the effects of injury on IL-12 production in humans. With the recent availability of a reliable immunoassay for IL-12, we undertook an investigation of IL-12 production by circulating monocytes/macrophages at multiple time points after injury in a series of patients with major trauma or burns. We also were interested to see how the production of IL-12 was correlated with the production of the antiinflammatory Th2 type cytokine, IL-10.

We had previously shown in a mouse model of burn injury that IL-12 therapy given for several days early after injury would increase survival after a septic challenge, cecal ligation and puncture (CLP).[13] Such treatment was associated with increased production of the proinflammatory cytokine interferon-gamma (IFNγ) and diminished production of IL-4. In the present studies, we wished to determine whether IL-12, given on alternate days in low doses, which corresponded to IL-12 regimens known to be minimally toxic in human clinical trials, would have a similarly beneficial effect on resistance to infection in the mouse burn model. Moreover, we wished to assess the safety and efficacy of low-dose IL-12 treatment carried out beyond the time of septic challenge. We were further interested in learning what effect such a treatment regimen would have on the production of the proinflammatory cytokines IFNγ and tumor necrosis factor-alpha (TNFα) and on the production of the antiinflammatory cytokine IL-10. Inhibition of the latter cytokine with a specific monoclonal antibody had been reported to be detrimental to survival in a similar animal model of sepsis.[16]

PATIENTS AND METHODS

Patient Population

Twenty-seven adult patients, 11 of whom had major traumatic injury (Injury Severity Score, 27–57)[1,7,10] and 16 of whom had major burns (25–95% body surface area), were studied at multiple time points after injury after obtaining informed consent. The study was approved by the Brigham and Women's Hospital Committee for the protection of Human Subjects from Research Risks and was in accordance with the National Institutes of Health guidelines. There were 25 men and 2 women in the study group, ages 18 to 56 years. Eighteen healthy human volunteers of both sexes, 21 to 55 years of age, served as control subjects.

Human Peripheral Blood Mononuclear Cells

Twenty- to 30-mL samples of venous blood were withdrawn from patients at intervals from 1 to 40 days after injury on 53 occasions. A similar blood sample was drawn simultaneously from a control subject on each occasion. As described previously,[14] peripheral blood mononuclear cells (PBMCs) were collected by centrifugation of heparinized blood on Ficoll-Hypaque (Pharmacia, Piscataway, NJ) for 35 minutes at 400*g*. The interface cells were collected, washed three times in complete medium (minimal essential medium with antibiotics [penicillin, streptomycin, and amphotericin B], 2 mmol/L glutamine, 1% HEPES buffer, and 5% fetal bovine serum [FBS], all reagents from Gibco/BRL, Grand Island, NY), and counted with trypan blue to determine viability and Turk's stain for observations of morphology. The cells were always more than 95% viable. Because patient interface cells sometimes had considerable contamination with the immature myeloid cells, the percentage of mononuclear cells was estimated by Turk's stain and cell

Supported in part by NIH grant R01GM3563314, a grant from Genetics Institute, Andover, MA, the David Brook Fund for Vascular Research, the Julian and Eunice Cohen Surgery Fund, and a grant from Werein zur Foerderung der Fortbildung und Forschung in der Anaesthesie, Wurzburg, Germany.

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Accepted for publication April 5, 1999.

counts were adjusted accordingly so that the total number of mononuclear cells per tissue culture was similar in each experiment.

Human PBMC Stimulation by Lipopolysaccharide

Two hundred microliters containing 1×10^5 PBMCs was placed in each well in 96-well tissue culture plates (NUNC, Rothskilde, Denmark) in complete medium and were cultured at 37°C in 5% CO_2 with or without the addition of lipopolysaccharide (LPS, *Escherichia coli* 055:B5; Difco, Detroit, MI), 0.3 μg/well. The supernatants were removed at 24 hours, and similar wells were pooled and frozen at −20°C until tested.

Human Adherent Cell Stimulation by LPS

Two hundred microliters of complete medium containing PBMCs at 5×10^6 cells/mL was allowed to adhere to each well of a flat-bottomed tissue culture plate (NUNC) for 1 hour at 37°C in 5% CO_2. Nonadherent cells were removed by washing the plates three times in RPMI-1640 medium (Gibco) with additions as described above but without FBS, and the adherent cells were cultured for 24 hours in 200 μL RPMI-1640 medium in the presence of 0.3 μg/well LPS or with no additions. The supernatants were removed at 24 hours, and similar wells were pooled and frozen at −20°C until tested.

Mouse Burn Model

Eight-week-old male A/J mice obtained from Jackson Laboratories (Bar Harbor, ME) were acclimatized for 1 week in the animal facilities at the Harvard Medical School. Animals were maintained with water and mouse food ad libitum. Care of the animals and all of the procedures performed were in accordance with National Institutes of Health guidelines, with the permission of the Harvard Medical School Standing Committee on Animals. Mice were studied in groups of 5 to 15.

After induction of anesthesia with intraperitoneal pentobarbital (66 μg/g body weight), the animals were shaved over the dorsum and placed in a specially constructed plastic mold that exposed approximately 25% of total body surface area. The mold containing the animal was immersed in 90°C water for 9 seconds to produce burn injury and in room-temperature water for sham burn. The procedure has been shown to result in a histologically proven full-thickness burn of the exposed dorsum.[17] After injury, animals were resuscitated immediately with 1 mL 0.9% sterile saline per mouse intraperitoneally.

Cecal Ligation and Puncture

As described previously,[17] mice were anesthetized and the abdomen was shaved and opened in the midline. The cecum was delivered and ligated near its base with a 4-0 silk ligature. The cecum was punctured twice with a 25-g needle and was then replaced in the abdomen. The wound was closed with 5-0 nylon. Animals again were resuscitated with 1 mL 0.09% saline intraperitoneally.

IL-12 Treatment

In some experiments, mice were treated with recombinant murine IL-12 (donated by Dr. Stanley Wolf of Genetics Institute, Andover, MA) at varying doses in 0.2 mL saline administered intraperitoneally on an alternate-day regimen beginning on the day of injury or sham injury. Control animals were treated with saline vehicle only.

At day 10, the time of maximum mortality from an infectious challenge in this model,[17] some groups of IL-12-treated animals underwent cecal ligation and puncture (CLP) and were observed for survival for at least 10 days. In some experiments, the IL-12 treatment was continued through day 9, the day before CLP. In other experiments the therapy was continued through to day 11 beyond CLP.

Cytokine Production by Mouse Spleen Cells

Mice, 5 to 10 per group, were killed in a CO_2 chamber on day 10 or day 13 after burn or sham burn injury. The spleens were removed and teased apart with forceps. Capsules were discarded and cells were harvested from plates with several washings to ensure complete recovery. The splenocytes were washed in RPMI-1640 medium containing HEPES buffer and antibiotics as above and resuspended in RPMI-1640 complete medium containing L-glutamine, HEPES buffer, 2-mercaptoethanol, antibiotics, and 5% FBS. The cells were washed, counted, and resuspended in RPMI-1640 complete medium, and 200 μL containing 5×10^5 cells was placed in each well of a 96-well tissue culture plate in the presence or absence of antimouse CD3 monoclonal antibody (145-2C11, protein A purified, 1 μg/mL). Plates were cultured for 48 hours at 37°C in 5% CO_2. The supernatants were then collected for determination of cytokine production. Spleen cells in suspension at 1×10^7 cells/mL of complete medium were also allowed to adhere to each well of flat-bottom tissue culture plates for 1 hour at 37°C in 5% CO_2. Nonadherent cells were removed by washing the plates three times in RPMI-1640 medium with additions as described above but without FBS, and the adherent cells were cultured for 48 hours in 200 μL RPMI-1640 medium in the presence of 0.3 μg/well LPS or with no additions. The supernatants were removed at 48 hours, and similar wells were pooled and frozen at −20°C until tested.

Cytokine Immunoassays

Mouse and human IL-12 p70 levels were measured by sandwich enzyme-linked immunoadsorbent assays

(ELISA). In the human assay, the capture antibody was a mouse antihuman IL-12 p70 monoclonal antibody (MAB611) and the detection antibody was a biotinylated goat antihuman IL-12 monoclonal antibody (BAF219) (both antibodies from R&D Systems, Minneapolis, MN). The capture antibody, diluted to 4 μg/mL in 0.1 mol/L carbonate buffer, pH 9.6, was added to 96-well ELISA plates (NUNC). The plates were incubated overnight at 4°C and then washed three times in wash solution (phosphate-buffered saline plus 0.05% Tween 20). Active sites on the plates were blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 hour at room temperature, and the plates again were washed three times in wash solution. The samples and standard (R&D Systems), diluted in medium corresponding to the samples, were added in 50-μL volumes to each well. The plates were incubated for 60 minutes at 37°C in 5% CO_2 and washed three times, and 50 μL of the detection antibody, diluted to 0.3 μg/mL in wash solution containing 0.1% bovine serum albumin, was added to each plate. Plates were incubated for 60 minutes at room temperature and washed three times as before. Fifty microliters of avidin-alkaline phosphate conjugate (Sigma, St. Louis, MO), diluted 1:2,000 in wash solution with 0.1% bovine serum albumin, was added to each well, and the plates again were incubated for 1 hour at room temperature. Plates were washed six times, the substrate paranitrophenylphosphate disodium, 1 mg/mL in substrate diluent (pH 9.8, 0.05 mol/L sodium carbonate buffer with 1 mmol magnesium chloride), was added at 100 μL per well, and the plates were incubated at room temperature until acceptable color development occurred. The results were read at 450 nm in an ELISA reader (UV Max, Molecular Devices, Palo Alto, CA). Data were obtained from standard curves generated by ELISA software (Soft Max, Molecular Devices).

The ELISA for murine IL-12 p70 was purchased as a kit from Genzyme (Boston, MA). The manufacturer's guidelines were followed for all assays. ELISAs for mouse and human IL-10 mouse IFNγ, and mouse TNFα have been previously described.[7–14] All immunoassays were tested for sensitivity and specificity using recombinant cytokines (Genzyme). To avoid interassay variation, all supernatants were tested for IFNγ, IL-10, and TNFα in the same assay.

Statistical Analysis

Control subjects versus burn and trauma patients and sham burn mice versus burn mice were compared regarding cytokine production by the Mann-Whitney test or Welch's *t* test. Survival of animals after CLP was compared by the Wilcoxon-Gehan test. The level of significance was $P < .05$.

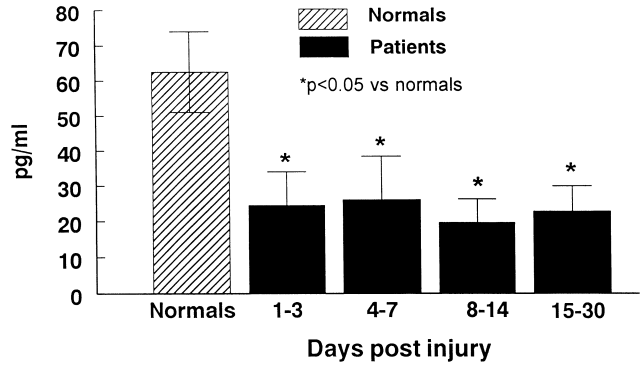

Figure 1. Interleukin-12 p70 secretion by lipopolysaccharide-stimulated adherent cells from the peripheral blood mononuclear cells of 27 burn and trauma patients measured by enzyme-linked immunoadsorbent assay on 53 occasions at serial intervals after injury and compared with 18 normal control subjects. The patients' adherent cells produced significantly less interleukin-12 than cells from normal persons at multiple intervals beginning early after injury.

RESULTS

IL-12 Production by Patients' Adherent PBMCs

The 27 burn and trauma patients were studied on 53 occasions. Adherent cells were stimulated with LPS and IL-12 production was measured in culture supernatants at 24 hours by ELISA. On each occasion, IL-12 production by a patient's adherent cells was compared with that of a control subject. Burn and trauma patients were indistinguishable in their IL-12 production after injury and were therefore grouped together.

Figure 1 shows that even at the early (1–3 days) interval after injury, patients' adherent cells produced less IL-12 after LPS stimulation than did those of normal persons. IL-12 production tended to remain suppressed in the patients at all intervals studied and reached a nadir between 8 and 14 days after injury. Failure of patient adherent cells to produce IL-12 in the first 14 days after injury did not appear to correlate with subsequent development of sepsis or systemic inflammatory response syndrome, which occurred in 19 of the 27 patients.

Because IL-12 and IL-10 are known to have generally opposite effects, with IL-12 being largely proinflammatory and IL-10 antiinflammatory, we were interested to see whether IL-12 and IL-10 production had a reciprocal relation in our patient population. As we have previously reported, the production of IL-10 by LPS-stimulated human adherent cells has been consistently low in our laboratory.[14] However, IL-10 production by LPS-stimulated whole PBMC populations is ordinarily vigorous. Therefore, we made serial observations in 18 patients on 38 occasions for IL-10 production by LPS-stimulated PBMCs and observations in 8 of these patients for IL-12 production by the same PBMC cultures on 21 occasions. Patients were compared with simultaneously studied normal subjects. Figure 2

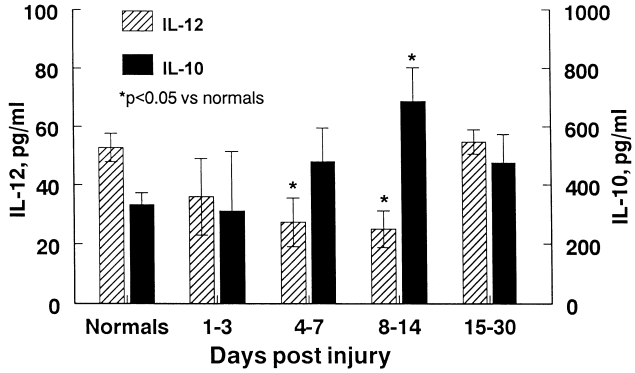

Figure 2. Production of interleukin-12 (IL-12) and IL-10 by lipopolysaccharide-stimulated peripheral blood mononuclear cells (PBMCs) of burn and trauma patients at serial intervals after injury. Thirty-eight observations of IL-10 production were made in 18 patients and 21 observations of IL-12 production in 8 of the same patients. PBMC IL-12 production was significantly diminished in the 8- to 14-day interval after injury when compared with simultaneously studied normal subjects. At the same interval, IL-10 production by the patients' PBMCs was significantly elevated.

shows that 8 to 14 days after injury, IL-12 production by LPS-stimulated patient PBMCs was markedly lower than that of PBMC from normal subjects; at the same time interval, IL-10 production was significantly elevated over that of the normal population. In these experiments, patient PBMC IL-12 production was not diminished in the 15- to 30-day interval, in contrast to the findings with adherent cells noted above. This discrepancy may have resulted from the fact that PBMCs contain a mixed cell population, compared with the more homogeneous adherent cell population.

IL-12 Production by Mouse Splenic Adherent Cells

Although we had previously reported reduced IL-12 production by mouse splenic adherent cells at 10 days after burn injury,[7] as assessed by a bioassay, we thought it appropriate to confirm these findings using ELISA. Therefore, groups of 8 to 10 burn and sham burn mice were killed at serial intervals after burn injury, and LPS-stimulated IL-12 p70 production by adherent splenocytes was measured by ELISA. In rough agreement with the patient studies reported above, IL-12 production by burn mouse adherent cells began to diminish by day 5 after injury, reaching a nadir at day 7, when burn splenocyte IL-12 production was 3.9 pg/mL versus 13.5 pg/mL for sham burn mice. The ratio of burn to sham IL-12 production was significantly lower at that time ($P < .05$) compared with day 1.

Effect of IL-12 Therapy on Survival After CLP

We had previously reported that a brief course of recombinant murine IL-12 therapy at moderate doses given after burn injury and before CLP would markedly increase the survival of burn animals subjected to CLP on day 13, the time of maximum mortality from CLP in this model. Because the effects of IL-12 in vivo are proinflammatory and removal of IL-10, a principal antiinflammatory mediator, with anti–IL-10 antibody treatment increases the death rate after CLP in mice,[16] we thought it might be harmful to continue IL-12 therapy up to the time of CLP or beyond. However, it also was apparent that if IL-12 therapy were ever to prove clinically useful in seriously injured patients, the safety of IL-12 given up to and beyond the time of infectious challenge needed to be assessed.

We therefore set out to explore the efficacy of lower-dose IL-12 treatment administered on a clinically relevant alternate-day basis beginning at the time of burn or sham burn injury. Burn and sham burn animals were studied in groups of 12 to 15. All animals received alternate-day intraperitoneal injections of recombinant murine IL-12 in doses of 5 or 10 ng or saline vehicle. One of the groups receiving 5 ng IL-12 also received alternate-day injections of indomethacin (1 mg in 0.2 mL saline), because prior experiments had indicated that indomethacin had a synergistic effect with cytokines in increasing survival after CLP.[17] All animals underwent CLP on day 10 and were observed for survival for at least 14 days.

Figure 3 shows that the 10-ng dose of IL-12 had no effect on the survival of sham burn animals. The 5-ng dose of IL-12 administered to burn animals increased survival to that of sham burn controls. The 10-ng dose was modestly efficacious, and the addition of indomethacin to the 5-ng dose regimen clearly produced inferior results.

Because the alternate-day 5-ng dose appeared to increase survival markedly after CLP in burn animals, the efficacy of this dosage regimen was tested when the IL-12 treatment was carried through day 11, 24 hours after the septic challenge with CLP. Again, the burn animals treated with 5 ng IL-12 had a survival equivalent to that of sham burn animals

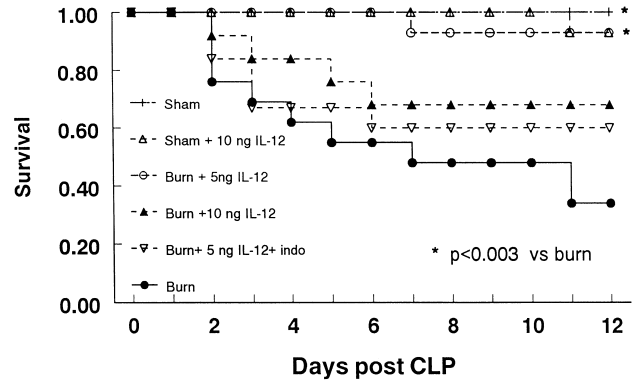

Figure 3. Survival of groups of 12 to 15 burn and sham burn mice after cecal ligation and puncture performed 10 days after injury. Burn mice receiving 5 ng interleukin-12 (IL-12) every other day for 9 days beginning on the day of injury had a survival similar to that of sham burn mice. IL-12 in a 10-ng dose appeared to have a less favorable effect on survival, and the addition of indomethacin to the 5-ng dose of IL-12 produced inferior results. IL-12 treatment did not affect the survival of sham burn mice.

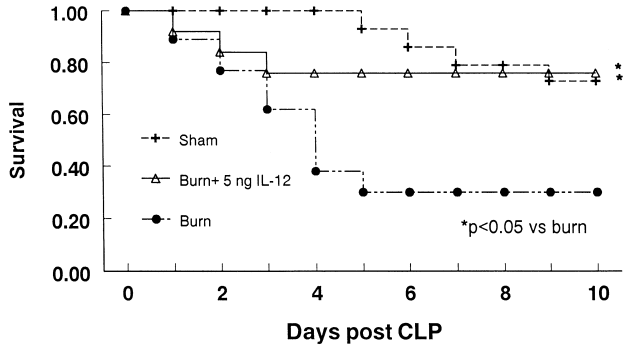

Figure 4. Survival of groups of 12 to 15 burn and sham burn mice after cecal ligation and puncture performed on day 10 after injury. The burn mice treated with 5 ng interleukin-12 (IL-12) every other day had a survival similar to that of sham burn controls, even though the IL-12 treatment was continued through day 11, beyond the time of cecal ligation and puncture. Untreated burn animals had the expected high death rate.

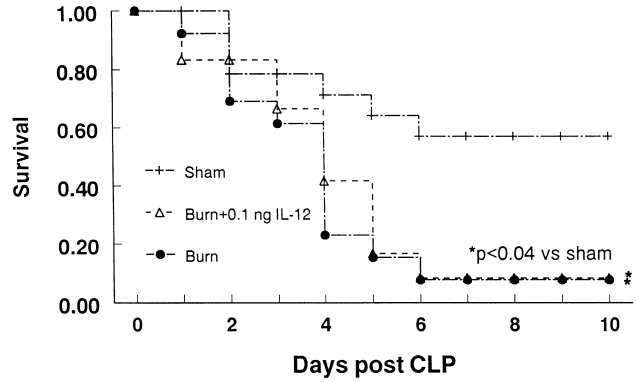

Figure 6. Survival of groups of 12 to 15 burn and sham burn mice after cecal ligation and puncture performed on day 10 after injury. The dose of 0.1 ng interleukin-12 (IL-12) given every other day and continued beyond the time of cecal ligation and puncture had no beneficial effect on the survival of burn animals.

(Fig. 4), whereas the saline-treated burn animals had the anticipated high death rate.

To determine the lowest effective dose of IL-12, further experiments were carried out with doses of 1 ng and 0.1 ng carried beyond the time of CLP. Figures 5 and 6 show that the 1-ng dose was as effective as the 5-ng dose in increasing survival after CLP, whereas the 0.1-ng dose had no effect. It therefore appeared that low doses of IL-12 given on an alternate-day basis markedly improved survival after a septic challenge in burn animals.

Effect of IL-12 Treatment on Cytokine Production by Mouse Splenocytes

Because of the apparently beneficial effect of alternate-day low-dose IL-12 therapy on survival, even when carried beyond the time of CLP, we thought it appropriate to determine the effect of such treatment on proinflammatory and antiinflammatory cytokine production. Burn and sham burn mice were studied in groups of 5 to 10. The animals were treated with 5 ng IL-12 every other day or with saline vehicle alone. They were killed on day 10, at the time CLP would ordinarily have been performed. Cytokine production by splenocytes stimulated with anti-CD3 monoclonal antibody or by splenic adherent cells stimulated with LPS was studied by ELISA.

Figure 7 shows that IL-12 therapy primed burn and sham splenocytes for increased production of IFNγ compared with splenocytes from saline-treated burn and sham animals. As shown in Figure 8, LPS-stimulated adherent splenic cells were primed to produce markedly more TNFα in the IL-12–treated burn group than in any of the other groups of mice. Finally, as shown in Figure 9, anti–CD3-treated splenocytes from burn animals produced markedly more IL-10 than those from sham burn controls. IL-12–treated sham animals had no increase in IL-10 production, whereas IL-12–treated burn animals showed a further elevation in IL-10 secretion ($P < .05$ vs. untreated burn animals).

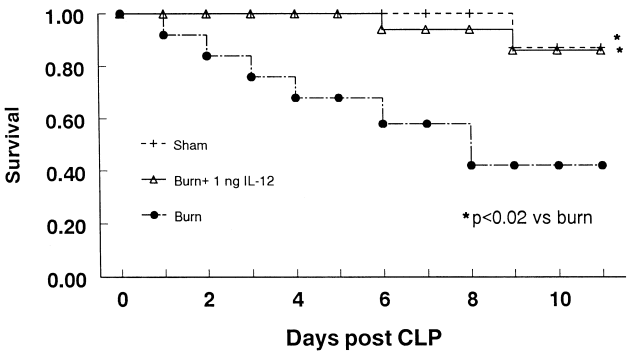

Figure 5. Survival of groups of 12 to 15 burn and sham burn mice after cecal ligation and puncture performed on day 10 after injury. The dose of 1 ng interleukin-12 (IL-12) given every other day in burn animals and continued beyond the time of cecal ligation and puncture produced survival equivalent to that of sham burn controls.

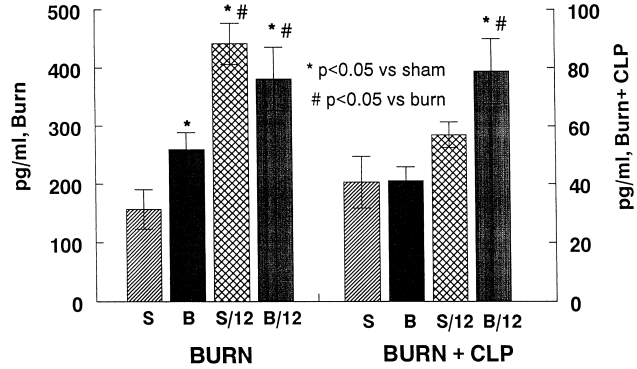

Figure 7. Interferon-gamma production by anti-CD3 antibody-stimulated splenocytes from groups of 5 to 10 burn and sham burn animals 10 days or 13 days after injury (after cecal ligation and puncture on day 10). The burn groups treated with interleukin-12 (IL-12) produced significantly more interferon-gamma than untreated animals. S and S/12 indicate untreated and IL-12–treated sham mice. B and B/12 indicate untreated and IL-12–treated burn mice.

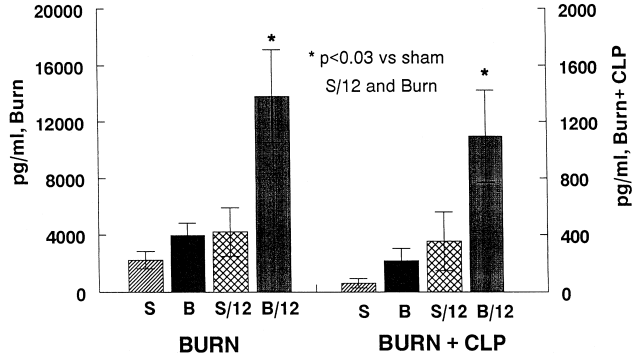

Figure 8. Tumor necrosis factor-alpha production by lipopolysaccharide-stimulated adherent splenocytes from groups of 5 to 10 burn and sham burn animals 10 days or 13 days after injury (after cecal ligation and puncture on day 10). Splenocyte production of tumor necrosis factor-alpha was markedly increased in the burn animals treated with interleukin-12.

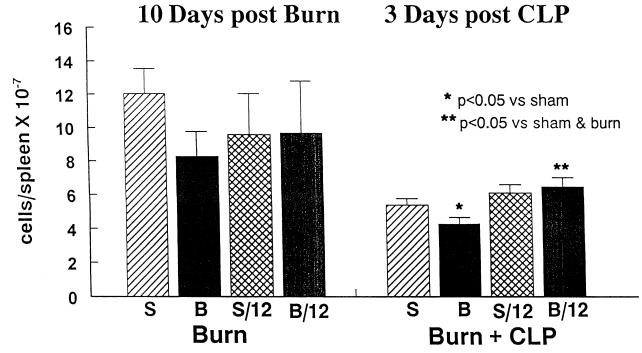

Figure 10. Total mononuclear cells per spleen in groups of five burn and sham burn animals at day 10 after the burn and at day 13, 3 days after cecal ligation and puncture was performed on day 10. The mononuclear cell population in the spleen was markedly reduced 3 days after cecal ligation and puncture, and treatment with interleukin-12 significantly increased the splenic mononuclear cell population of burn animals.

In a further experiment, groups of five burn and sham burn animals were treated with either 5 ng IL-12 or saline control in an alternate-day regimen as before. Animals on this occasion were subjected to CLP on day 10 and then were killed 3 days after CLP, on day 13. Spleens were again harvested and cultures of splenocytes or adherent cells were stimulated as before with either anti-CD3 monoclonal antibody or LPS. Again, production of IFNγ, TNFα, and IL-10 was determined by ELISA. As shown in Figure 7, the splenocytes from IL-12–treated burn animals were primed to produce significantly more IFNγ 3 days after CLP than splenocytes from saline-treated burn animals. IL-12 produced an insignificant increase in IFNγ production by sham splenocytes. As shown in Figure 8, splenic adherent cells from burn animals treated with IL-12 produced markedly more TNFγ with LPS stimulation than adherent splenocytes from saline-treated burn animals. IL-12 treatment modestly but not significantly increased sham animals' TNFα secretion. IFNγ and TNFα production by all groups was markedly lower after than before CLP.

Finally, as shown in Figure 9, stimulated IL-10 production was increased in all groups 3 days after CLP. However, the significantly higher IL-10 production seen in the saline-treated burns was increased further by IL-12 therapy. IL-12 treatment in the sham group had no apparent effect on stimulated IL-10 production 3 days after CLP. In contrast to IFNγ and TNFα production, IL-10 production was increased, not decreased, after CLP.

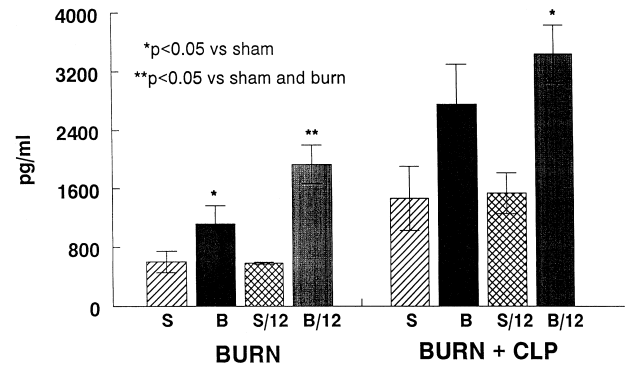

Figure 9. Production of interleukin-10 (IL-10) by anti-CD3–stimulated splenocytes harvested from groups of 5 to 10 burn and sham burn mice before cecal ligation and puncture on day 10 after injury or on day 13 (after cecal ligation and puncture on day 10). IL-12 treatment increased IL-10 production by burn splenocytes, both before and after cecal ligation and puncture. Splenocytes from untreated burn animals produced more IL-10 than sham burn splenocytes at both intervals.

Effect of Low-Dose IL-12 Therapy on Splenic Cellularity

In an attempt to delineate the effects of low-dose IL-12 therapy, we again studied burn and sham burn mice in groups of five. Animals received 5 ng IL-12 or saline vehicle every other day and were killed at day 10 or had treatment continued through day 11, 1 day after CLP. The latter groups were killed at day 13. Spleens were again removed and cell counts per spleen were determined in a hemocytometer. Figure 10 shows that there was a nonsignificant decrease in cellularity in the spleens of saline-treated burn animals at day 10. After CLP, there was markedly decreased cellularity in the spleens of all groups. Burn animals showed a significant reduction over sham burn animals. IL-12 therapy increased cellularity of burn spleens. It thus appeared that IL-12 therapy increased splenic cellularity in addition to increasing stimulated production of IFNγ, TNFα, and IL-10.

DISCUSSION

During the past 4 years, we and others have reported evidence that in humans and in experimental animals, serious injury is associated with a loss of function and of cytokine production by the Th1 subset of lymphocytes,

whereas Th2 function and cytokine production are apparently maintained or increased.[4–11] Because IL-12 is known to be the principal agent that induces naive T-helper cells to assume the Th1 phenotype,[15] an examination of the effect of injury on IL-12 production appeared to be warranted. The present results indicate that serious traumatic or burn injury in humans and major burn injury in the mouse is followed by a diminished capacity to produce IL-12 by monocytes/macrophages from the peripheral blood in humans and from the spleen in the mouse model. The diminished capacity to produce IL-12 by the usual cellular sources of this cytokine, even when maximally stimulated by bacterial LPS, may explain at least in part the apparent inability to maintain Th1 function and cytokine production that has been noted in several clinical and animal studies approximately 1 week after serious injury.[2–4,7–9] The present findings are also in concert with those of a recent report showing diminished IL-12 production in patients in whom sepsis developed after gastrointestinal surgery.[18]

The present studies have also demonstrated a reciprocal relation between stimulated IL-12 production and IL-10 production in the PBMC population of injured patients. In two clinical studies, one from our laboratory, increased production of IL-10 after injury was found to be associated with an increased risk of infection.[10,14] In animal studies, IL-10 has been shown to play an early role in the diminished Th1 function seen after injury,[11] possibly by its effect on decreasing production of IL-12 and other cytokines by monocytes/macrophages or dendritic cells. In contrast, there is evidence that IL-10 plays a beneficial role in sepsis.[16,19] Of particular relevance to the present animal studies, one report has shown that treatment with anti–IL-10 monoclonal antibody begun at the time of CLP in the mouse significantly increases the septic death rate.[16] Thus, prophylactic treatment with anti–IL-10 antibody does not appear likely to be efficacious in seriously injured patients.

However, with the present demonstration of markedly diminished IL-12 production induced by serious injury, the possibility of prophylactic treatment with exogenous IL-12 to prevent diminished resistance to infection seen in patients after major thermal or traumatic injury has some attractions. IL-12 is a potent inducer of IFNγ production by both T cells and natural killer cells.[15] In turn, IFNγ is a potent activator of monocyte/macrophage bactericidal activity and stimulates production of cytokines, which activate other cells important in host defenses, including polymorphonuclear leukocytes. In addition, IFNγ is known to play an essential role in initiating the production of complement fixing antibodies of the IgG2 class.[11]

We had previously reported that early treatment of burn mice with moderate doses (25–50 ng) of recombinant murine IL-12 markedly increased survival of the burn-injured animals after CLP performed at day 10 after injury,[13] the time of maximum mortality from this septic challenge.[17] However, treatment in these prior experiments was stopped well before CLP because of fear of an excessive proinflammatory response after the onset of sepsis. Nevertheless, it seemed likely that if IL-12 therapy were ever to prove useful clinically, its safety and efficacy would need to be demonstrated even when treatment was continued up to and even beyond the onset of sepsis. Consequently, in the present work we studied the effects of lower doses of IL-12 administered in a clinically relevant alternate-day regimen carried up to and, in some studies, beyond the time of CLP. We found that over a log of IL-12 dosages (1–10 ng/mouse), IL-12 dramatically improved survival of burn animals subjected to CLP. In several experiments, the survival of IL-12–treated burn animals was equal to that of sham burn controls. The 1- and 5-ng IL-12 dosages, which were highly efficacious in this model, would be roughly equivalent to 1- to 5-μg doses in adult patients, which are respectively below or just above the threshold dose shown to produce side effects in prior human trials.[20]

To attempt to explain the efficacy of IL-12 therapy in the burn mouse model, we looked at the effect of this treatment regimen on the cellularity and cytokine production in a central lymphoid organ, the spleen, before and after septic challenge. There was a modest but not significant diminution in cellularity of burn versus sham burn spleens just before CLP; IL-12 therapy restored burn spleen cellularity to levels slightly above those of sham burn animals. Three days after CLP, cellularity was markedly decreased in all study groups, including sham burn, burn, IL-12–treated sham burn, and IL-12–treated burn animals. Again, saline-treated burn cellularity was lowest and IL-12 therapy raised burn cellularity to levels modestly above other groups.

IL-12 therapy had much more dramatic effects on cytokine production. As anticipated, IL-12 markedly increased stimulated IFNγ production, both before and after the onset of sepsis. TNFα production was also markedly increased by IL-12 treatment. In contrast, IL-10 production, as previously shown,[14] was dramatically increased in splenocyte cultures from burn animals, and this was maintained or further increased by IL-12 therapy, both before and after the onset of sepsis. These results suggest that IL-12 therapy may be efficacious because it increases the production of the proinflammatory cytokines IFNγ and TNFα, which stimulate host defenses, and at the same time increases or maintains the production of IL-10, thus ensuring a balance between proinflammatory and antiinflammatory mediators.

In summary, we have shown in the present studies that the capacity to produce IL-12 by cells of the monocyte/macrophage lineage is significantly reduced after serious injury in humans and an animal model. There appears to be a reciprocal relation between diminished IL-12 production and increased IL-10 production at approximately 1 week after injury. Finally, low-dose IL-12 therapy in an animal burn model markedly increases survival after a septic challenge, even when treatment is carried beyond the onset of sepsis. Studies of cytokine production at the time of septic challenge and 3 days thereafter demonstrated that low-dose IL-12 treatment increased production of proinflammatory

mediators important in host defense and at the same time maintained or increased production of IL-10, an important antiinflammatory cytokine.

Acknowledgments

The authors thank Dr. Stanley Wolf of Genetics Institute for his generous gift of murine IL-12 and for invaluable advice, and Ms. Sally Bemis for expert preparation of the manuscript.

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